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Sensitive Detection of Chromosome Copy Number Aberrations in Prostate Cancer by Fluorescence *In Situ* Hybridization

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The pattern of chromosomal aberrations and their significance in prostate cancer are poorly understood. We studied 23 prostate cancer and 10 benign prostatic hyperplasia (BPH) specimens by fluorescence* in situ *hybridization (FISH) using pericentromeric repeat-specific probes for 10 different chromosomes. The aims of the study were: 1) to compare the sensitivity of FISH and DNA flow cytometry in aneuploidy detection, 2) to determine which chromosome copy number changes are most common, and 3) which probe combinations would be most effective in aneuploidy diagnosis. Disaggregated tumor cells from formalin-fixed, paraffin-embedded tissues were pretreated with our newly developed method based on hot glycerol solution to improve probe penetration. All BPH specimens were diploid by DNA flow cytometry and showed no numerical chromosome aberrations by FISH. In prostate cancer, flow cytometry showed abnormal DNA content in 35% of cases, whereas 74% were abnormal by FISH. Aberrant copy number of chromosomes 8 (48% of cases), X (43% of cases), and 7 (39% of cases) were most common. Ninety-four percent of all aneuploid cases would have been detected with these three probes alone. Simple chromosome losses were uncommon but in DNA tetraploid tumors relative losses (trisomy or disomy) of several chromosomes were often found, suggesting progression of prostate cancer through tetraploidization followed by losses of selected chromosomes. In conclusion, our results indicate that FISH using three selected chromosome-specific probes is two to three times more sensitive than flow cytometric DNA content analysis in aneuploidy detection. (Am J Pathol 1994, 145:624–630)

Prostate cancer is the most common cancer among men in the United States and the second most common in Finland.[1,2] Despite of the high prevalence, relatively little is known of the genetic changes underlying the development of this malignancy, the biological role of these changes, and their possible diagnostic usefulness. In classical cytogenetic studies of primary prostate cancers, the vast majority of tumors only show a diploid male (46, XY) karyotype.[3–7] The typical 10 to 30% frequency of aneuploidy found in these studies may reflect the poor growth of aneuploid prostatic carcinoma cells *in vitro*.[8] In contrast, more than half of prostate cancers have aneuploid DNA content, according to flow and image cytometric measurements.[9]

Fluorescence *in situ* hybridization (FISH) makes it possible to analyze chromosomal abnormalities from interphase tumor nuclei[10,11] thereby avoiding problems due to selection of cells during *in vitro* culture. Chromosome copy number changes have often been detected in breast and bladder cancer[12–14] as well as in prostate cancer[15–21] using probes that recognize chromosome-specific repeat sequences, such as α-satellite DNAs. However, comprehensive studies with many different probes have not been conducted in prostate cancer, and the diagnostic value and biological significance of the aberrations found by FISH are poorly characterized.

Because prostate cancer specimens are routinely fixed in formalin and embedded in paraffin, we have

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developed a method that allows reliable FISH analysis of nuclei disaggregated from routinely fixed paraffin-embedded tumor specimens.[22] Here, we used this technique to study 10 paraffin-embedded benign prostatic hyperplasia (BPH) and 23 prostate cancer specimens with pericentromeric repeat probes for chromosomes 1, 3, 7, 8, 10, 16, 17, 18, X, and Y. The primary aims of the study were to: 1) compare the sensitivity of FISH and DNA flow cytometry in the diagnosis of aneuploidy, 2) determine which chromosome copy number changes are most common, and 3) determine the most effective probe combinations for large-scale clinical studies of prostate cancer.

Materials and Methods

Patients

Twenty-three paraffin-embedded prostate cancer specimens from patients who underwent radical prostatectomy in the Tampere University Hospital between 1988 and 1992 were used in this study. The clinical data were collected from patient records. The patients were staged, according to the TNM classification,[23] and tumors graded according to World Health Organization recommendations.[24] Two patients had stage T1N0M0, 11 T2N0M0, 2 T2N1M0, 2 T3N0M0, 2 T3N1M0, 1 T4N0M0, and 3 TXN0M0 (T stage not known). Ten paraffin-embedded BPH specimens were also analyzed.

Probes

Probes specific for the pericentromeric repeat regions of chromosomes 1 (1q12 [pUC177]), 3 (D3Z1 [pα3.5]), 7 (D7Z1 [p7αtet]), 8 (D8Z2 [pJM128]), 10 (D10Z1 [pBS609-51 and pA10RP8]), 16 (D16Z3 [pHUR195]), 17 (D17Z1 [p17H8]), and 18 (D18Z1 [p18R]) were biotinylated using nick translation (BioNick kit; GIBCO BRL, Gaithersburg, MD). Fluorophore-labeled chromosome enumerator probes for chromosomes X (SpectrumCEP X, SpectrumOrange) and Y (SpectrumCEP Y, SpectrumGreen) were obtained from Imagenetics (Imagenetics, Framingham, MA).

Preparation of Nuclei from Paraffin-Embedded Tumors

The most representative paraffin-embedded tumor blocks were selected by histopathological examination of hematoxylin and eosin-stained slides. Nuclei were isolated from one or two 50-μmol/L sections using a modification of the method described by Heider et al.[25] Briefly, the sections were deparaffinized with xylene, rehydrated in an ethanol series, and placed in 1 ml of Carlsberg solution (0.1% Sigma protease XXIV, 0.1 M Tris, 0.07 M NaCl, pH 7.2) for 1 hour at 37 C. Half of the nuclear suspension was used for DNA flow cytometry, whereas the other half was pipetted on Vectabond-treated (Vector Laboratories, Burlingame, CA) slides (5 to 10/specimen) and air-dried.

DNA Flow Cytometry

Nuclei were stained with 50 μg/ml ethidium bromide, followed by RNase A1 treatment (1 mg/ml) filtered through a nylon net and analyzed by an Epics-C flow cytometer (Coulter Electronics Inc., Hialeah, FL). Methods and criteria for the analysis and interpretation of the DNA histograms have been described previously.[26] DNA aneuploidy was considered to be present if two clearly separate peaks were found in the DNA histogram (DNA index >.05). The coefficient of variation (±SD) of the diploid G0/G1 peak was 8.5 ± 1.4%.

Fluorescence In Situ Hybridization

FISH was performed as described in detail elsewhere.[22] Before FISH, the slides were pretreated by heating in 50% glycerol/0.1X standard saline citrate (SSC), pH 7.5 (1X SSC is 0.15 M NaCl, 0.015 M Na citrate), solution at 90 C for 3 minutes to decondense the chromatin and to improve hybridization efficiency. The slides were denaturated in 70% formamide/2X SSC (pH 7) at 74 C for 5 minutes, dehydrated in an ethanol series, and treated with 8 μg/ml proteinase K (Sigma Chemical Co., St. Louis, MO) in a 20 mmol/L Tris/2 mmol/L $CaCl_2$ buffer at 37 C for 7.5 minutes followed by dehydration. A 10-μl volume of hybridization mixture consisting of 55% formamide/2X SSC, 10% dextran sulfate, 0.5 μg unlabeled carrier DNA (sonicated herring sperm DNA), 62 ng Cot-1 DNA (GIBCO BRL), and 5 ng of labeled probe was denaturated at 70 C for 5 minutes and applied on slides.

Hybridization was conducted under a coverslip for 48 hours at 37 C. After hybridization, the slides were washed three times in 50% formamide/2X SSC at 45 C and once in 4X SSC at room temperature. Immunostaining was conducted with avidin-FITC (Vector Laboratories), followed by biotinylated goat antiavidin antibody and another layer of avidin-FITC.[22] After staining, slides were mounted in an antifade solution

(Vectashield; Vector Laboratories) containing propidium iodide as a DNA counterstain. Dual color hybridization with directly conjugated CEP probes for chromosomes X and Y was conducted, according to manufacturer's instructions after the slide pretreatment described above. The slides were counterstained with 4,6-diamidino-2-phenylindole in an antifade solution.

A Nikon SA epifluorescence microscope (Nikon Corporation, Tokyo, Japan) was used to determine signal copy numbers from a minimum of 100 nuclei per hybridization. Previously published guidelines[11] were used as scoring criteria. Briefly, only nuclei that were intact and nonoverlapping and showed signals of approximately the same intensity were scored. Spots in paired arrangement (split spots) were counted as one signal.

Nuclei isolated from paraffin-embedded normal lymph nodes were used as controls. In these samples, the mean ± SD percentage of nuclei with more than two signals (or more than one signal for chromosomes X and Y) per nucleus was 2.6 ± 1.9%. The mean ± SD percentage of nuclei with one signal (or zero signals for chromosomes X and Y) was 6.7 ± 3.8%. Based on these experiments, tumors were considered trisomic or tetrasomic (or disomic for X and Y) if more than 10% of nuclei showed three or four signals (or two signals with probes for X and Y) and monosomic (or nullisomic for X and Y) if more than 20% of nuclei showed only one signal (or no signal with probes for X and Y).

Statistical Analyses

Statistical analyses of the data were conducted using the BMDP Statistical Software Package.[27] The associations of chromosome copy numbers and other parameters were evaluated either by Pearson χ^2 test, Fisher's exact test (BMDP4F), or by nonparametric Kruskal-Wallis test (BMDP3S).

Results

Chromosome Copy Number Aberrations in Prostate Cancers and BPH

No chromosomal copy number changes were observed in 10 BPH specimens by FISH. Fluorescence signal distributions in BPH were similar to those of normal lymph node samples (Figure 1). In contrast, one or more nondisomic cell populations were found in 17 (74%) prostate cancers (Figure 2, Table 1). The number of aberrant chromosomes per tumor ranged from 1 to 10. The most frequent aberrations, mostly gains (trisomy or tetrasomy), affected chromosomes 8 (48%), X (43%), and 7 (39%). Increased copy number of chromosomes 1, 10, 17, and Y were also found in more than 30% of cases. Monosomy was only found in two cases, tumor 1 (for chromosomes 10 and 16) and 13 (for chromosome 7). Figure 3 shows a comparison of aneuploidy frequencies with each of the probes alone and the cumulative frequency. Two probes (for chromosomes 8 and X) would have detected 82% and three (for chromosomes 8, X, and 7) 94% of all aneuploid cases.

Comparison of FISH Results with DNA Aneuploidy

All BPH samples had a diploid DNA content by DNA flow cytometry, whereas eight (35%) prostate cancers

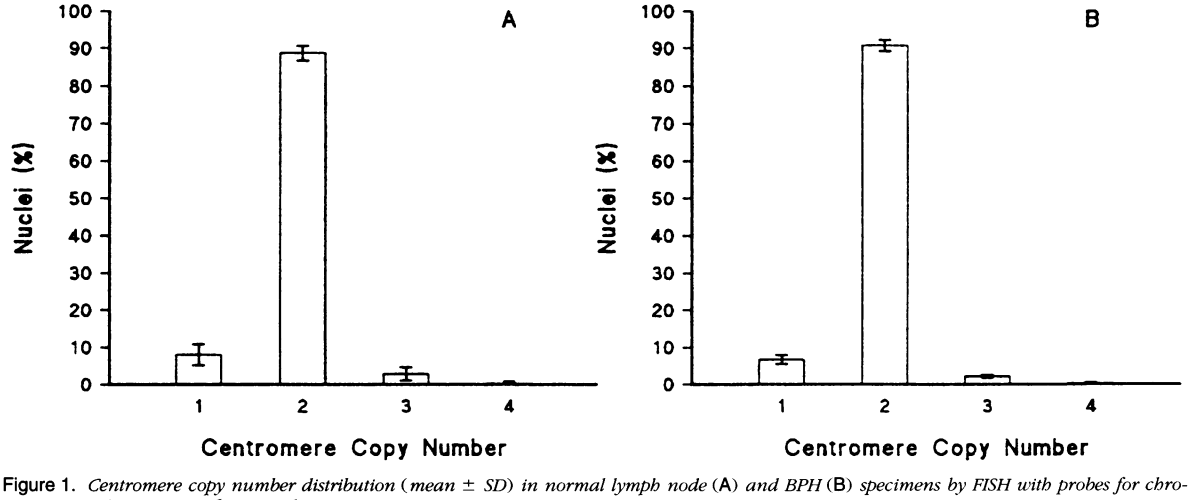

Figure 1. *Centromere copy number distribution (mean ± SD) in normal lymph node* (A) *and BPH* (B) *specimens by FISH with probes for chromosomes 1, 3, 7, 8, 10, 16, 17, and 18.*

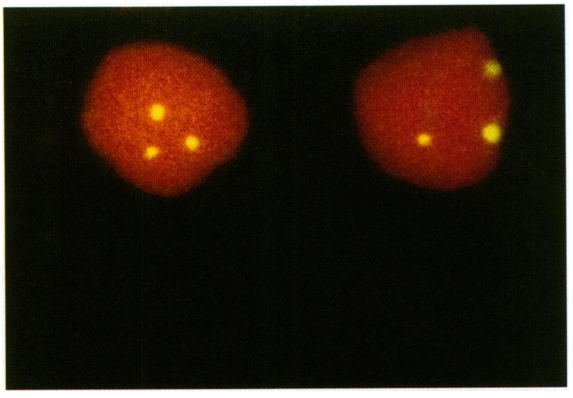

Figure 2. *A photomicrograph of a prostate tumor after FISH with a probe for the pericentromeric region of chromosome 1. Nuclei were counterstained with propidium iodide (original magnification ×600).*

showed DNA aneuploidy. All DNA aneuploid cases were near tetraploid (DNA index 1.97 to 2.12) by flow cytometry and showed increased chromosome copy number by FISH with at least five different probes. However, not all chromosomes in the DNA tetraploid cancers showed four copies by FISH. Figure 4 represents an example of the relative loss of chromosomes in a DNA tetraploid tumor. This tumor shows four copies of chromosome 8, three copies of chromosome 17, and two copies of chromosome 18 as the predominant cell clone. Overall, relative losses of chromosome 18 were more often found (50%) in the DNA tetraploid tumors than losses of any other chromosomes. Chromosome copy number aberrations were also found in nine tumors that had diploid DNA content by flow cytometry. Only one to three chromosomes were affected in these tumors.

Comparison of FISH Results with Tumor Proliferative Activity and Histological Grade

Mean S phase fraction was 5.3 ± 2.3% in the DNA diploid and 7.0 ± 1.8% in the DNA tetraploid prostate cancers ($P = 0.07$). The association between chromosome copy number changes and S phase fraction was most significant for chromosome 1 ($P = 0.010$), followed by that of chromosomes 10 ($P = 0.047$), 17 ($P = 0.048$), and Y ($P = 0.052$). Gain of chromosome 10 was associated with high histological grade ($P = 0.013$).

Discussion

This study on interphase cytogenetics of 23 prostate cancer and 10 hyperplasia specimens was conducted with probes for 10 different chromosomes, thereby providing a more comprehensive view of the chromosome aberrations than previous studies limited to two to six probes.[15–21] This is reflected in the high frequency of numerical aberrations found in this study (74%) in tumors that represented clinically early stage prostate cancer. Previous FISH studies with fewer probes have reported prevalences ranging from approximately 36 to 66% in uncultured primary tumors[16,17,21] to 90% in short-term cultures.[15] Overall, all these frequencies are higher than the percentage of clonal numerical chromosomal aberrations (13 to 37%) found in primary prostate cancers by classical cytogenetic methods.[3–7] This clearly illustrates the enhanced potential of interphase FISH studies in avoiding the selection that takes place in the preparation of metaphase cells from primary tumors.[8] All benign hyperplasia specimens showed only disomic cells with every chromosome probe indicating that chromosome copy number changes seen in prostate cancer may be associated with the progression to malignancy and thus have diagnostic significance.

Less than half of the aneuploid tumors detected by FISH were found by flow cytometric DNA content measurements. This indicates how DNA flow cytometry, especially when performed from paraffin-embedded tumors, cannot distinguish aneuploid cell clones with only few numerical chromosomal changes from diploid clones.[28] With a single probe (for chromosome 8), FISH would have detected more aneuploid cases (48%) than DNA flow cytometry (35%) and by adding two more probes (for chromosomes X and 7), 70% of tumors would have shown aneuploidy. This represents 94% of tumors that showed changes with all 10 probes. Thus, the vast majority of aneuploid tumors could be identified in a single three-color FISH analysis targeting these three selected chromosomes.[29] Significant improvements in aneuploidy detection rate beyond this three-probe combination can only be achieved by using a very large selection of probes, possibly involving chromosomes other than the 10 studied here. It should also be recognized that detecting the highest frequency of aneuploidy may not be as important as finding those chromosome changes that are most strongly associated with the disease course.

The most frequent chromosome copy number aberration in our study was the gain of chromosome 8 (48%), supporting the findings of Macoska et al[21] who found gain of chromosome 8 in 54% of prostate cancers. Molecular genetic studies[21,30] and our own recent findings with comparative genomic hybridization (TV et al, unpublished observations) have shown an association between the gain of the long arm of chro-

Table 1. *Chromosome Copy Number in 23 Prostate Cancers According to FISH Analysis*

Tumor Id	TNM	Grade	DI	Signal Copy Number of Major Cell Populations: Chromosomes 1	3	7	8	10	16	17	18	X	Y
1	100	I	1.00	2	2	2	3	1	1	2	2	1	1
2	200	I	1.00	2	2	2	2	2	2	2	2	1	1
3	200	II	1.00	2	2	2	2	2	2	2	2	1	1
4	100	I	1.00	2	2	3	2	2	2	3	2	1	1
5	210	II	1.00	2	2	2	2	2	2	2	2	1	1
6	200	I	1.00	2	2	2	2	2	2	2	2	1	1
7	200	II	1.00	2	2	2	3	2	2	2	2	1	1
8	200	II	1.00	3	2	2	2	2	2	2	4	2	1
9	210	II	1.00	2	2	2	2	2	2	2	2	1	1
10	X00	II	1.00	2	2	2	3, 4	2	2	2	2	1	1
11	200	I	1.00	2	2	2	2	2	2	2	2	1	1
12	X00	II	1.00	2	2	3	2	2	2	2	2	2	1
13	300	II	1.00	2	2	1	2	2	2	2	2	1	2
14	100	I	1.00	2	2	2	4	2	2	2	4	2	1
15	400	II	1.00	2	2	2	2	2	3	2	2	1	1
16	300	II	1.97	4	3, 4	3	3	2	2	3, 4	4	2	2
17	200	II	2.00	3	2	3	3	3	2	3, 4	3, 4	2	2
18	200	II	2.00	3, 4	3, 4	3, 4	4	2	2	3	3, 4	2	2
19	310	III	2.00	2	3	2	2	3	3, 4	2	2	2	2
20	310	III	2.01	3	4	4	3, 4	3, 4	2	3	2	2	2
21	X00	III	2.02	3, 4	2	2	4	3, 4	3, 4	3	2	1	1
22	200	II	2.05	3	3	4	3, 4	4	3, 4	3, 4	2	2	2
23	200	I	2.12	3, 4	3, 4	3, 4	3, 4	3	3, 4	4	3	2	2

DI, DNA index as defined by flow cytometry. Tumor was considered trisomic and/or tetrasomic (or disomic for chromosomes X or Y) if more than 10% of cells showed three or four signals (or two signals with chromosomes X or Y), respectively. Tumor was considered monosomic if more than 20% of cells showed only one signal.

mosome 8 and the deletion of 8p in prostate cancer. This same pattern of 8p loss and 8q gain is also found in breast cancer (AK et al, unpublished observations). This suggests that centromeric probe hybridizations may in this case reflect important underlying structural chromosome aberrations.

Pure monosomic cell populations were only found in two cases. Previous cytogenetic studies have reported that the loss of chromosome Y is the most common karyotypic change in prostate cancer.[3,5–7] This has also been suspected to be a culture artifact because chromosome Y is sometimes lost from short-term cultures of normal tissues.[31,32] Although we found no cases with total loss of Y, three tetraploid tumors (16, 20, and 22) showed relative loss of Y in a subpopulation of cancer cells as indicated by the presence of nuclei with two signals for X and only one for Y in a dual color hybridization. Besides the loss of Y, relative losses of other chromosomes were also common in tumors with a tetraploid DNA content. Chromosome 18 was most often affected (50%). In the evaluation of relative chromosome losses, it is important to exclude the effects of poor hybridization efficiency. Control hybridizations to normal lymph nodes and BPH specimens never showed evidence of this. Based on the finding of only two signals by FISH in more than 90% of cells, in some tumors with tetraploid DNA content, also tends to exclude poor hybridization efficiency (Figure 4, C). Furthermore, the specimens studied with each of the probes came from the same nuclear preparation. Thus, intratumor heterogeneity can be excluded.

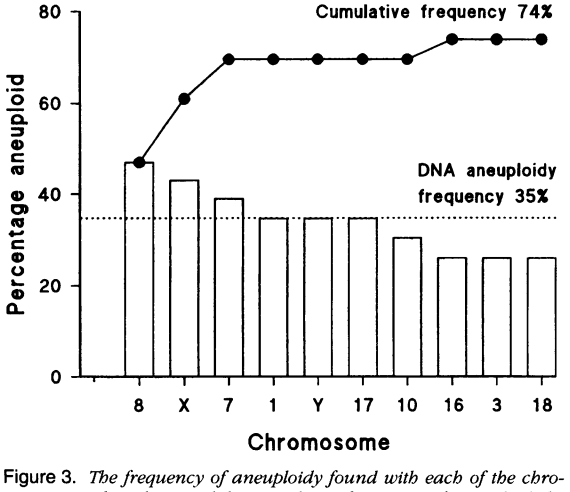

Figure 3. *The frequency of aneuploidy found with each of the chromosome probes alone and the cumulative frequency of aneuploidy by combining the probes. The frequency of DNA content aneuploidy found by flow cytometric analysis is shown as a reference.*

The lack of simple monosomic populations but the frequent presence of relative chromosome losses in the DNA tetraploid tumors is consistent with the concept of cytogenetic evolution of cancer by tetraploidization followed by losses of selected chromosomes.[33] Flow and image cytometric measurements of DNA ploidy level may therefore provide a useful

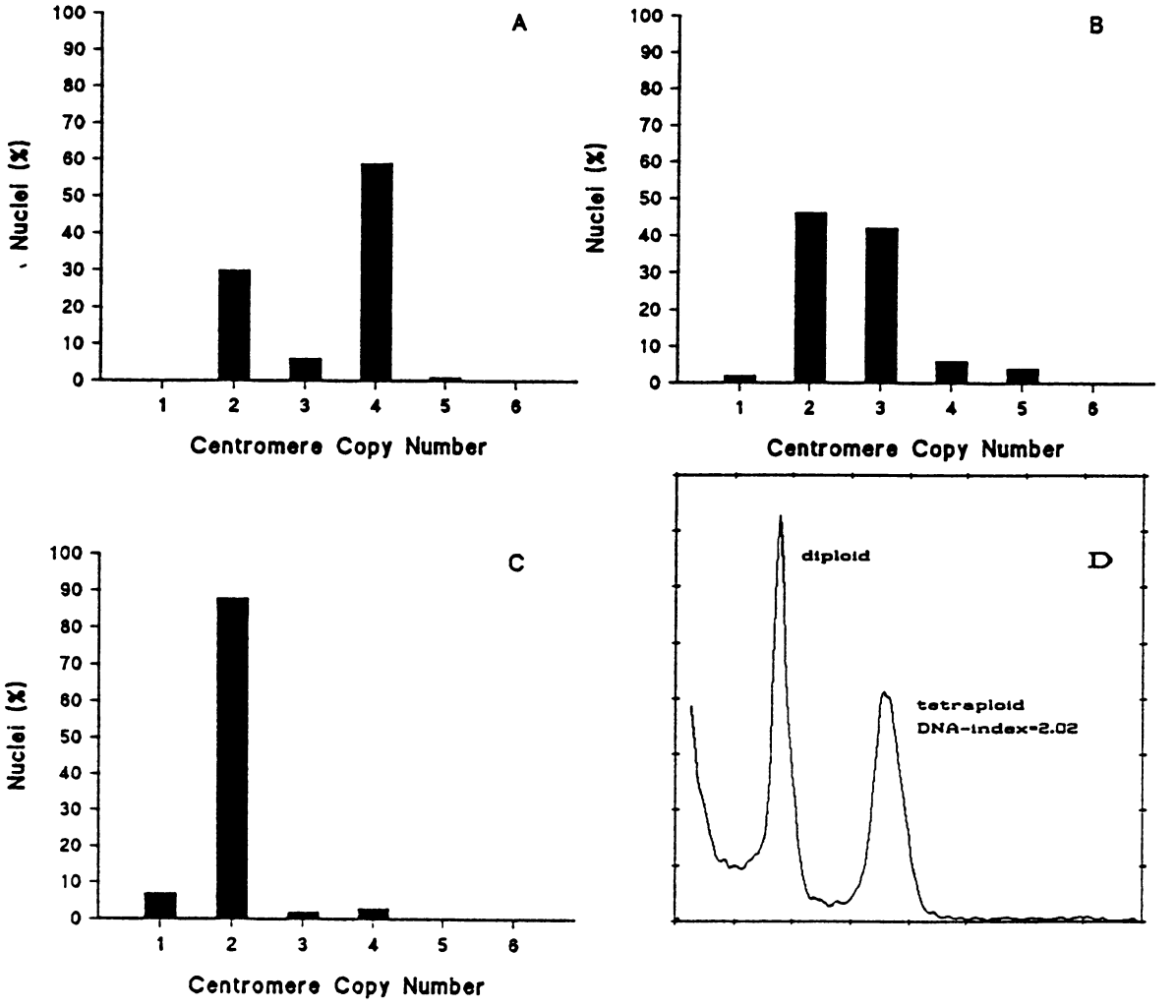

Figure 4. *An example of a prostate cancer that was tetrasomic for chromosome 8* (A), *trisomic for chromosome 17* (B), *and disomic for chromosome 18* (C). *DNA flow cytometric analysis indicated that 51% of cells had a tetraploid DNA content* (D).

adjunct to the interpretation of FISH data. The analogy in classical cytogenetics is the definition of chromosome aberrations in reference to the modal chromosome number in each specimen. For example, chromosome trisomies most likely have a different biological significance if they occur in a DNA diploid tumor compared with a DNA tetraploid tumor.

In a preliminary study of clinicopathological associations of the aberrations found by FISH, gain of chromosome 1 was more significantly associated with high S phase fraction than DNA aneuploidy. The biological consequences of the individual chromosome aberrations may vary from chromosome to chromosome and should be studied in more detail. Aneuploid DNA content by flow cytometric analysis shows prognostic value in prostate cancer.[9] Because the frequency of aneuploidy by FISH is higher and the involvement of the different chromosomes varies from one tumor to another, it will be interesting to study the prognostic value of aberrations detected by FISH.

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